Medical PROGRESS

Refer to: Gross SR: Animal models of glycogen storage conditions—Their relation to human disease (Medical Progress). West J Med 123:194-201, Sep 1975

Animal Models of Glycogen Storage Conditions

Their Relation to Human Disease

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THIS ARTICLE will briefly review the human glycogenoses and the mechanisms that regulate tissue glycogen concentrations. Biochemical studies of human glycogenoses will then be examined in light of recent studies on two animal models of glycogen storage conditions.

Human Glycogenoses

Although phosphorylase was originally proposed to be the enzyme responsible for both glycogen synthesis and degradation,[1] it is now clear that there are separate pathways for synthesis and degradation (Figure 1). Mutations in the enzymes of the synthetic pathway would not be likely to produce glycogen storage. One exception is the deficiency in the enzyme catalyzing the formation of the branch points in glycogen, commonly called brancher enzyme (Table 1). Persons with this mutation do not necessarily have an accumulation of glycogen in their tissues, but their glycogen has an abnormal structure.[8,9] The pathologic changes occurring in this syndrome are thought to result from the abnormal glycogen structure, but the exact mechanism is uncertain.[8,9] The remaining glycogenoses (Table 1) result from an altered regulation of glycogenolysis.

In the degradative pathway, two enzymes act directly on glycogen (Figure 1). Phosphorylase catalyzes the phosphorolytic cleavage of glycogen to glucose l-phosphate, and debrancher enzyme hydrolyzes glycogen at branch points to glucose (Figure 1). Since glycogen is a highly branched polysaccharide and phosphorylase does not act on the branch points, the absence of either enzyme activity results in glycogen storage (Table 1).

Phosphorylase exists in two interconvertible forms, designated as *b* and *a*, that differ in their content of phosphate groups on specific serine residues.[10] The conversion of phosphorylase *b* to *a* is catalyzed by phosphorylase kinase (Figure 1) and the *a* to *b* conversion by phosphorylase phosphatase. Phosphorylase kinase also exists in a nonactivated, dephosphorylated form and an activated, phosphorylated form. The conversion of phosphorylase kinase to the activated form is catalyzed by adenosine-3′,5′-monophosphate (cyclic AMP)-dependent protein kinase, which also can phosphorylate glycogen synthase-I (independent of glucose 6-phosphate) to convert it to the nonactivated D form (dependent on glucose 6-phosphate for activity, Figure 1).[11] Deficiencies in the activity of phosphorylase kinase or cyclic AMP-dependent protein kinase have also been reported to produce glycogen storage disease in humans (Table 1).[12,13]

Two glycogenoses are associated with a deficiency in utilization of the products of glycogenolysis. One affects the liver whose glycogen content is an important source of blood glucose. The absence of hepatic glucose 6-phosphatase

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Supported in part by United States Public Health Service Grant GM 20412 and by a grant from the Muscular Dystrophy Associations of America.

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ABBREVIATIONS USED IN TEXT

ATP = adenosine triphosphate
AMP = adenosine monophosphate
Cyclic AMP = adenosine-3′,5′-monophosphate
G6-PD = glucose 6-phosphate dehydrogenase

activity blocks the conversion of glucose 6-phosphate to glucose, resulting in hypoglycemia and hepatic glycogen storage (Table 1). The products of glycogenolysis are also an ubiquitous source of substrate for the Embden-Meyerhof pathway and hence, for energy production. A deficiency of phosphofructokinase activity, a rate-limiting step in the Embden-Meyerhof pathway,[14,15] has been found to produce glycogen storage in skeletal muscle.[15] Tissue differences in the structure of phosphofructokinase may account for the limitation of this deficiency primarily to skeletal muscle.[15,16]

The remaining glycogenosis listed in Table 1 is associated with a decreased acid maltase activity.[17] In the classical presentation of this condition (Pompe's disease), death occurs in childhood.[9] Recently, less severe cases have been diagnosed in persons in the second to fifth decade of life.[18] The exact role of acid maltase in controlling tissue glycogen concentrations is not well understood (see Hers and van Hoof[17] for a further discussion).

Glycogenoses in Experimental Animals

There are only two glycogen storage conditions in experimental animals that have been extensively studied. These are deficiencies of liver glucose 6-phosphatase[19] and of skeletal muscle phosphorylase kinase,[20] both in the common house mouse, Mus musculus. The reasons for the lack of animal models of glycogenoses are undoubtedly multiple. A decreased glycogenolytic capability would be deleterious to survival in the wild, and lack of overt, pathognomonic features as seen, for example, in muscular dystrophies would mitigate against the ready recognition of such variants in animal colonies.

The glucose 6-phosphatase deficiencies were discovered among radiation-induced lethal mutations in X-irradiated mice.[19] Mice homozygous for the defective glucose 6-phosphatase die within several hours of birth from hypoglycemia.[19] Six different variants have been described that result in a glucose 6-phosphatase deficiency, and these variants are all alleles at the locus for albinism. In normal mice there is a pronounced rise in hepatic glucose 6-phosphatase within hours of birth; this rise fails to occur in the mutants.[19,21] Whether the glucose 6-phosphatase deficiencies in mice result from similar genetic changes as those reported in humans is uncertain.

The phosphorylase kinase deficiency mutation, on the other hand, is less deleterious. It is a single gene, sex-linked mutation (carried in I strain mice) associated with an elevation in skeletal muscle glycogen concentrations to three to five times that of control strains.[22] Affected animals have apparently normal muscle function.[20,22] This lack of a notably deleterious effect may be explained by the observation that fasting, exercise and even catecholamine administration stimulate glycogenolysis in skeletal muscle of I strain mice.[20,23] Early studies on this mutation found no significant phosphorylase kinase activity in I strain skeletal muscle extracts. Furthermore, no phosphorylase *b* to *a* conversion was observed in I strain skeletal muscle after epinephrine administration to the mice or electrical stimulation of isolated muscles to produce tetanic contractions, conditions that produce pronounced phosphorylase *b* to *a* conversion in skeletal muscle of control strains.[20,24] It was postulated that stimulation of phosphorylase *b* activity is the primary mechanism of action of glycogenolytic stimuli on I strain skeletal muscle.[22,24] Such a mechanism could be analogous to that postulated for hypoxic rat hearts in which a fall in tissue adenosine triphosphate (ATP) and glucose 6-phosphate concentrations and a rise in 5′-AMP and inorganic phosphate concentrations were observed with no increase in tissue phosphorylase *a* activity.[25] Since ATP and glucose 6-phosphate interact noncovalently with muscle phosphorylase *b* to inhibit its activity, and 5′-AMP and inorganic phosphate (which is also a substrate for the reaction) stimulate phosphorylase *b* activity,[26,27] the changes observed in hypoxic rat hearts indicated that stimulation of phosphorylase *b* activity would be an alternative mechanism to converting phosphorylase *b* to *a* for "turning on" glycogenolysis.[25] The early studies on the phosphorylase kinase deficiency mutation have been interpreted to indicate that the lack of a phosphorylase *b* to *a* converting mechanism was compatible with an essentially normal life.[28]

Findings on recent studies using more sensitive techniques, however, showed that skeletal muscle extracts of I strain mice do contain small amounts of phosphorylase kinase activity (0.3 percent of

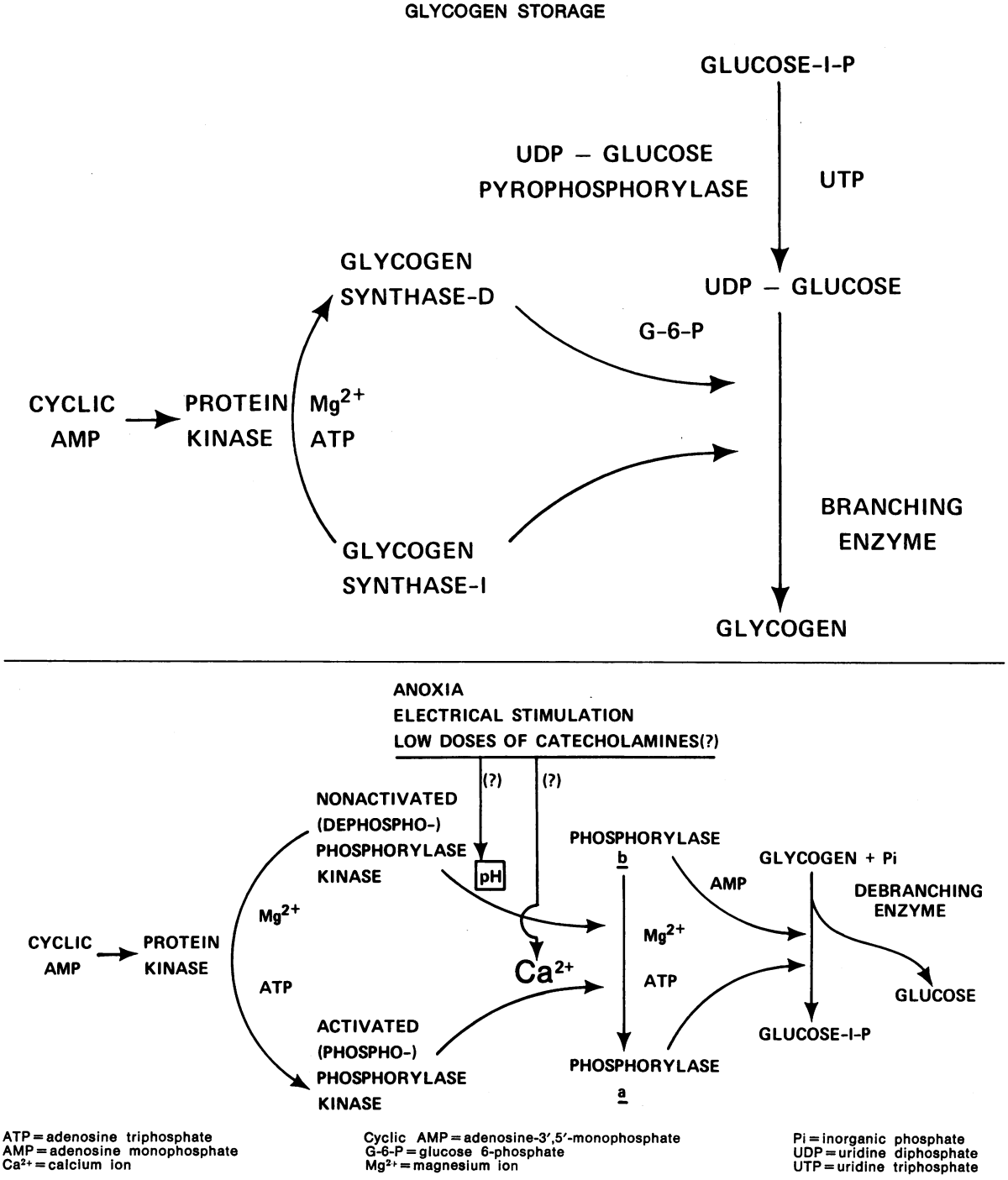

Figure 1.—Enzymatic pathways catalyzing the synthesis and degradation of glycogen. **Upper,** A detailed discussion of the regulation of glycogen synthesis may be found in Villar-Palasi and Larner.[2] **Lower,** The diagram of the glycogenolytic pathway is modified from Gross and Mayer.[3] In the glycogenolytic pathway experimental evidence has shown that anoxia, electrical stimulation and low doses of catecholamines control the catalytic activity of phosphorylase kinase in the absence of evidence for conversion of nonactivated to activated form.[4-6]

that of control strains),[29] as well as normal amounts of a protein that cross-reacts with antisera prepared against purified rabbit skeletal muscle phosphorylase kinase.[29,30] Incubation of I strain hemidiaphragms in oxygenated Krebs-Ringer bicarbonate buffer with either isoproterenol or epinephrine produced glycogenolysis, although the rate was less than that of control strain hemidiaphragms.[23,31] Catecholamines also were found to stimulate phosphorylase *b* to *a* conversion in I strain hemidiaphragms. There was a close correlation between the relative increase

TABLE 1.—*Human Glycogenoses*

Type	*Alternate Name*	*Enzyme Defect*	*Tissue Affected*	*Principle Clinical Features*
I	von Gierke's disease	Glucose 6-phosphatase	Liver, kidney, gut	Hepatomegaly, hypoglycemia, ketosis, acidosis
II	Pompe's disease	Lysosomal acid maltase (α-1,4-glucosidase)	Generalized	Cardiomegaly, heart failure, hepatomegaly, hypotonia; some adult cases with involvement of muscle and liver but without cardiomegaly
III	Limit dextrinosis	Debrancher enzyme (amylo-1,6-glucosidase)	Liver, heart, skeletal muscle	Moderate to marked hepatomegaly, none to moderate cardiomegaly; myopathies observed in some adults
IV	Amylopectinosis	Brancher enzyme (α-1, 4-glucan: α-1,4-glucan 6-glycosyl transferase)	Generalized	Hepato-splenomegaly, cirrhosis, ascites, liver failure
V	McArdle's disease	Muscle phosphorylase	Skeletal muscle	Temporary weakness and cramping of skeletal muscles after exercise, myoglobinuria
VI	Hers disease	Liver phosphorylase	Liver	Marked hepatomegaly
VII	....	Phosphofructokinase	Skeletal muscle, erythrocytes	Temporary weakness and cramping of skeletal muscle after exercise, pigmenturia
VIII	....	None yet demonstrated	Liver, brain	Hepatomegaly; neurologic deterioration progressing to decerebration and death
IX	....	Liver phosphorylase kinase	Liver	Marked hepatomegaly
X	....	Cyclic AMP-dependent protein kinase of skeletal muscle and presumably liver	Liver, skeletal muscle	Marked hepatomegaly

Adapted from Hug.[1]

in phosphorylase *a* activity and stimulation of glycogenolysis in I strain hemidiaphragms as compared with that of the control strain (C57BL).[23,31] These results suggest that catecholamines stimulate glycogenolysis in skeletal muscle of I strain, as in the control strain, by increasing phosphorylase *b* to *a* conversion. Future biochemical studies on human glycogenoses using more sensitive techniques may also indicate alternative interpretations of their biochemistry.

The genetic relationship of the phosphorylase kinase deficiency mutation in mice to those reported in humans is not clear. Huijing and Fernandes[32] reported a series of persons with a deficiency of phosphorylase kinase activity in leukocyte extracts. Of the 35 persons, 32 were male—suggesting that the defect is sex-linked, as is the murine phosphorylase kinase deficiency mutation.[22] Huijing and Fernandes[32] proposed that their subjects had a hepatic glycogenosis, although this was confirmed in only one case by assay of liver biopsy material. Consistent with their conclusion is the observation that leukocyte phosphorylase has properties similar to hepatic rather than skeletal muscle phosphorylase.[33] However, Hug and colleagues found that of their subjects who had a hepatic phosphorylase kinase deficiency confirmed by direct assay of biopsy material three of seven were females. Hepatic phosphorylase kinase activity is only slightly decreased in I strain mice to between 44 and 71 percent of that of control strains.[29,34]

Another complication is that studies of human hepatic glycogenoses have frequently failed to differentiate between deficiencies of phosphorylase and phosphorylase *b* to *a* converting activity.[12,17] Hepatic phosphorylase *b*, unlike that of muscle, is not fully active in the presence of saturating concentrations of 5′-AMP.[27] So to assay total activity in suspected glycogenoses, hepatic phosphorylase must be entirely converted to the *a* form by addition of phosphorylase kinase[12] or hepatic phosphorylase *b* may be assayed using 0.7 molar (M) sodium sulfate (Na_2SO_4) and 1 millimolar (mM) 5′-AMP.[35] Without an accurate determination of total phosphorylase activity, types VI and IX glycogenoses cannot be differentiated.

From the studies of glycogen storage conditions in mice several additional observations have been made that are relevant to the study of human glycogenoses.

• *To properly interpret biochemical studies of human skeletal muscle glycogenoses, it is necessary that control assays be carried out on muscle*

specimens matched for the age of the patient and the muscle type of the biopsy specimen.

While these precautions have been pointed out previously,[36,37] most studies still fail to report the age of the patient or muscle-type(s) used in control muscle samples. Illingworth[38] has argued that differences in enzyme activities between muscle types would not be of sufficient magnitude to be a factor in interpreting the results of biochemical studies of glycogenoses. However, when the enzymatic activities of the variant specimen are significantly above zero, the developmental state of the muscle and its type must be considered.

In Swiss-Webster mice at 6 to 8 days of age, the phosphorylase kinase activity of the gastrocnemius muscle was 12 percent of that of adults (Table 2), but even at 19 to 21 days of age, immediately before weaning, the activity was still only 38 percent of that of adults. Therefore, there was a slow postnatal increase in phosphorylase kinase activity in gastrocnemius muscle. In the diaphragm and soleus muscle the percent increase in phosphorylase kinase activity was less than in the gastrocnemius in the period examined. Total phosphorylase activity also slowly increased in developing mouse skeletal muscle (Table 2). Similar results on the development of skeletal muscle phosphorylase and phosphorylase kinase have been reported for the gastrocnemius of rats[40] and for abdominal and leg muscle of C57BL mice.[22] In rat liver there is a lesser postnatal increase in phosphorylase and phosphorylase kinase activity than in rat skeletal muscle.[40] The developmental patterns of the enzymes of glycogen metabolism have yet to be described in humans.

TABLE 2.—*Phosphorylase and Phosphorylase Kinase Activities of Three Skeletal Muscles of Young and Adult Mice*

Age	*Muscle*	*Phosphorylase Activity (units/gram)**	*Phosphorylase Kinase Activity (units/gram)†*
6-8 days . . .	gastrocnemius	9.2 ± 0.9	386 ± 34
	diaphragm	13.6 ± 1.5	332 ± 30
	soleus‡	8.0 ± 0.9	302 ± 24
19-21 days .	gastrocnemius	22.5 ± 3.7	1,259 ± 325
	diaphragm	13.5 ± 2.2	269 ± 20
	soleus	19.7 ± 3.9	1,031 ± 108
Adult	gastrocnemius	44.1 ± 4.5	3,282 ± 242
	diaphragm	17.7 ± 3.0	835 ± 136
	soleus	37.6 ± 4.9	1,440 ± 222

Skeletal muscle samples were homogenized in 10 volumes of 20 millimolar (mM) potassium fluoride (KF) and 4 mM ethylenediamine tetraacetic acid (EDTA), pH 7.4, and the 10,000×g (10 minutes) supernatant fraction was assayed for total phosphorylase activity in the presence of 2 mM 5′-adenosine monophosphate (AMP) and phosphorylase kinase activity at pH 8.2 as previously described.[29] Results are expressed as the mean ± standard error of the mean (SEM) of the values from four determinations on different litters at ages of 6 to 8 days and 19 to 21 days and from five determinations on adults (over 10 weeks of age). There was no statistically significant difference ($P>0.05$) in the protein contents of the supernatant fractions among the three age groups or among the three muscle types in adults.

*Unit = forms 1 μmole of glucose 1-phosphate per minute.[39]
†Unit = converts 1 unit of phosphorylase *b* to *a* per minute.[39]
‡At this age it was difficult to completely remove all of the gastrocnemius from the soleus sample so that the latter may be contaminated with the former.

The three muscles used in the study for Table 2 were selected because in rodents they represent three distinct types: fast twitch-white (gastrocnemius), fast twitch-red (diaphragm) and slow twitch-intermediate (soleus).[41,42] As Table 2 shows, the phosphorylase and phosphorylase kinase activities vary widely among the muscle types in adults with the diaphragm having activities of 40 percent and 25 percent, respectively, of that of gastrocnemius. These experiments show that under physiologic conditions murine skeletal muscle phosphorylase and phosphorylase kinase activities can vary up to ten fold depending on the age of the animal and the muscle type. Thus in biochemical studies of glycogenoses, control samples matched for age of the patient and muscle type are necessary for accurate interpretation of the data, unless the variant enzyme is devoid of activity.

• *Assays of mutant enzymatic activities* in vitro *under optimal conditions may not accurately correlate with* in vivo *activity.*

Skeletal muscle extracts of I strain gastrocnemius muscles convert purified rabbit skeletal muscle phosphorylase *b* to *a* at 0.3 percent of the rate of the enzyme from control strains (C57BL/St or Swiss-Webster) when assayed *in vitro* in dilute solution by conventional procedures.[29] Another system for measuring phosphorylase *b* to *a* conversion *in vitro* is the protein-glycogen complex isolated from skeletal muscle extracts by differential centrifugation.[10] Addition of ATP (10 mM), magnesium ion (Mg^{2+}) (50 mM), and calcium ion (Ca^{2+}) (4 mM) to the protein-glycogen complex isolated from Swiss-Webster mice produced conversion of 100 percent of the phosphorylase to the *a* form within 20 seconds.[29] In the I strain protein-glycogen complex under similar experimental conditions there was also a rapid phosphorylase *b* to *a* conversion, but only 11 percent phosphorylase *a* was pro-

duced.[29] This percent conversion of phosphorylase to the *a* form was the same as that observed in I strain diaphragms incubated with isoproterenol. So the phosphorylase *b* to *a* conversion in the protein-glycogen complex correlated more closely with the results in intact muscle than did the results of the conventional quantitative assays in dilute solution at apparently optimal conditions.[29]

A poor correlation of the enzyme deficiency measured at optimal assay conditions and the severity of the genetic disease has also been observed with several glucose 6-phosphate dehydrogenase (G6-PD) variants.[43] Patients with the Gd Union and Gd Mediterranean variants have G6-PD activities in their red blood cells of less than 5 percent of normal when assayed under optimal conditions and yet do not have chronic hemolytic anemia. This contrasts to subjects with the variants Gd Manchester and Gd Freiburg who have chronic hemolytic anemia but their G6-PD activities are 25 to 30 percent, and 10 to 20 percent of normal, respectively.[43] However, when these variant enzymes are assayed under simulated physiologic conditions (that is, with concentrations of substrates, activators and inhibitors found in red blood cells at pH 7.3), then the deficiencies in the enzyme activities correlate more closely with the severity of clinical disease.[43]

• *Multiple enzymatic deficiencies may occur as secondary effects of the primary lesion.*

Multiple deficiencies in enzymatic activities of glycogen metabolism have been frequently reported in human glycogenoses.[9,44] For example, there have been several persons found to be deficient in both debrancher enzyme and glucose 6-phosphatase activity.[8] The genetic implications of such multiple coinherited enzymatic deficiencies within any metabolic pathway are substantial and would argue strongly for an operon-type of genetic regulation in mammals. Alternative explanations are suggested from the results of studies in animals.

Secondary effects would result if the increased tissue glycogen concentration or deficiencies in the products of glycogenolysis altered the rate of synthesis or degradation of proteins. An apparent secondary effect of the phosphorylase kinase deficiency mutation is an increased total skeletal muscle phosphorylase activity (gastrocnemius) to a value of 60 percent greater than that of the C57BL strain.[29] The increase in total phosphorylase activity in I strain skeletal muscle may act to compensate for the decreased phosphorylase kinase activity. Mutations producing pronounced alterations in important metabolic pathways are likely to produce multiple secondary changes in the cellular physiology.[9]

Multiple enzymatic deficiencies could also result from mutations affecting a structural protein or altering a protein involved in the transduction of hormonal control of enzymes in the metabolic pathway. Studies on the mutations that produce a deficiency of hepatic glucose 6-phosphatase activity have shown multiple biochemical changes including notably decreased activities of tyrosine aminotransferase and serine dehydratase.[45] These mutants did not show a gene dosage effect for these abnormalities, that is, mice heterozygous for the mutant alleles could not be differentiated from those homozygous for the wild-type alleles. Furthermore, several of the mutant alleles showed complementation, that is, mice doubly heterozygous for certain combinations of the separate mutant alleles were viable and showed essentially normal neonatal hepatic glucose 6-phosphatase activities.[45] These results indicate that these alleles at the albino locus do not code for the structural gene for glucose 6-phosphatase.

Studies on ultrastructural changes in the glucose 6-phosphatase deficiency mutations have shown severe abnormalities of the hepatic endoplasmic reticulum membranes and Golgi apparatus.[46] Since glucose 6-phosphatase is localized in microsomal membranes, structural changes in these membranes could notably reduce its activity. However, neither hepatic tyrosine aminotransferase nor serine dehydratase is a microsomal enzyme, so that altered microsomal structure cannot entirely explain the multiple effects. The activities of all three enzymes can, however, be induced in normal mice by administration of hormones or by dietary factors, and cyclic AMP is thought to be a common intermediary in these enzyme inductions. Since these inductions do not occur in the mutant animals,[21,47] it has been suggested that an alteration in one of the proteins involved in the transduction of the hormonal response might be the primary defect.[47] Thus, there are several alternative explanations to an operon-like hypothesis for multiple enzymatic deficiencies in a metabolic pathway.

• *The lack of a cross-reacting protein to an antiserum produced to the normal enzyme does not necessarily show that the mutation results in the absence of the mutant enzyme in the tissue.*

Analyses of tissue extracts of mutations affecting enzymes frequently include a test for cross-reacting material to an antiserum prepared against the normal enzyme.[48,49] The presence of cross-reacting material indicates the presence of an enzyme with an altered structure. However, the lack of cross-reacting material does not necessarily show that there is an absence of a structurally altered enzyme. For example, tests for cross-reacting material in I strain skeletal muscle extracts were conducted with antisera prepared in goats and guinea pigs to purified rabbit skeletal muscle phosphorylase kinase. The antiserum prepared in guinea pigs cross-reacted with normal mouse phosphorylase kinase but not with I strain muscle extracts when tested by double diffusion in agar (Figure 2) or quantitative precipitation assays (Gross SR, Longshore MA and Pangburn S, unpublished observations). With goat antiserum, cross-reacting material was found in I strain extracts with both techniques (Figure 2).[29] It has now been recognized in several systems that antibodies may or may not bind antigens that are structurally related to the antigen.[50] Hence, mutant tissue extracts should be tested with several different antisera prepared against the normal enzyme in species that are not closely related. Only then is a lack of cross-reactivity in the mutant tissue likely to be significant. It is important for understanding the mechanisms regulating genetic expression in mammalian systems to differentiate between mutations that result in a complete absence of enzyme and those that result in an extensively modified protein.

Summary

The paucity of animal models of human glycogen storage conditions substantially restricts the use of experimental models to obtain information relevant to human disease. Studies on two such animal models with glycogen storage, the phosphorylase kinase and glucose 6-phosphatase deficiencies in mice, have indicated important principles that may be applicable to the elucidation of the biochemical and molecular bases of human glycogenoses and to their diagnosis.

- Control specimens should be matched for the age of patient and, if a skeletal muscle biopsy specimen, for the muscle type to allow accurate interpretation of the biochemical data obtained in biopsy material from patients with a glycogenosis.
- Enzymatic assay conditions *in vitro* should attempt to simulate conditions in intact tissue, especially with respect to concentrations of substrates and inhibitors. This also aids in the interpretation of the biochemical data.
- Changes in more than one enzymatic activity may be observed in affected patients. This may complicate both the diagnosis and attempts to un-

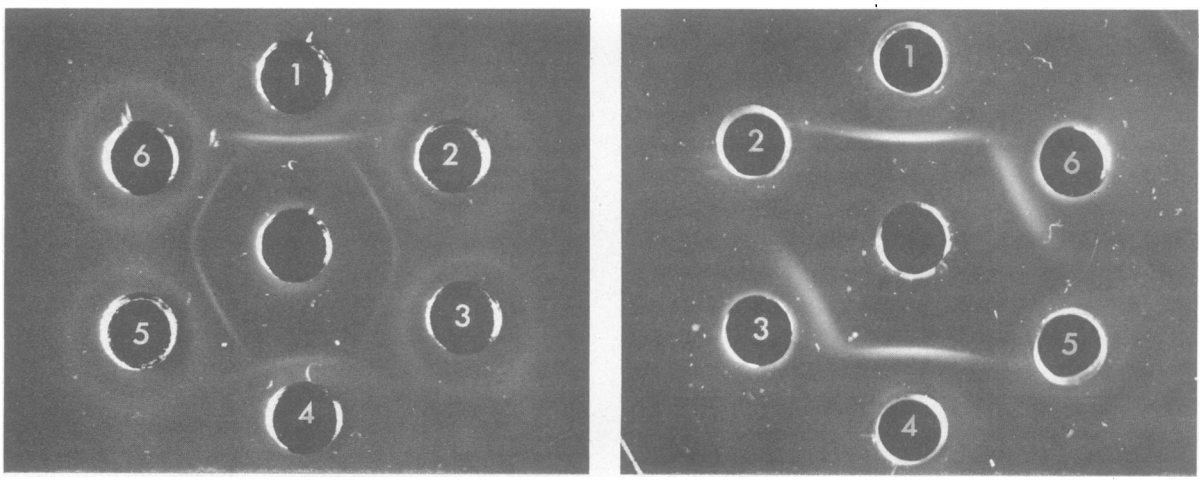

Figure 2.—Differential Cross-Reactivity of I Strain Phosphorylase Kinase with Antiphosphorylase Kinase Sera Prepared in Goats and Guinea Pigs. Ouchterlony double diffusion analysis was done by incubating the agar plates for 3 to 4 days in a humidified atmosphere at room temperature. **Center well, Left,** goat antiserum to rabbit muscle phosphorylase kinase; **Right,** guinea pig antiserum to rabbit phosphorylase kinase. **Outside wells 1** and **4,** rabbit muscle phosphorylase kinase (40 pellet fraction prepared as described by Krebs and co-workers[51]) 200 μg of protein except in **well 4** at left (100 μg); **2** and **5,** 40 pellet fraction from skeletal muscle of I strain mice,[29] 1 mg protein; **3** and **6,** 40 pellet fraction from skeletal muscle of Swiss-Webster mice,[29] 1 mg protein. The antisera were prepared to purified rabbit phosphorylase kinase (Peak II, with the method of Hayakawa and co-workers[52]) as previously described.[29,53]

derstand the nature of the primary defect. Multiple enzymatic deficiencies may occur where the primary mutation alters the cellular structure or proteins necessary for the transduction of hormonal signals. In addition, enzymatic activities may decrease or increase secondary to an alteration in cellular function that changes the rate of synthesis or degradation of multiple proteins.

- Immunochemical studies of tissue extracts from affected patients should use antisera made in several divergent species, if the results of the initial studies with a single antiserum show a lack of cross-reactivity. This would provide more substantial evidence to differentiate whether the mutant tissue extract lacks the mutant enzyme or has an extensively modified enzyme.

It is hoped that future studies on glycogen storage conditions in experimental animals will not only provide additional information on the points raised here but will also describe new areas of importance for the understanding of human glycogenoses.

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